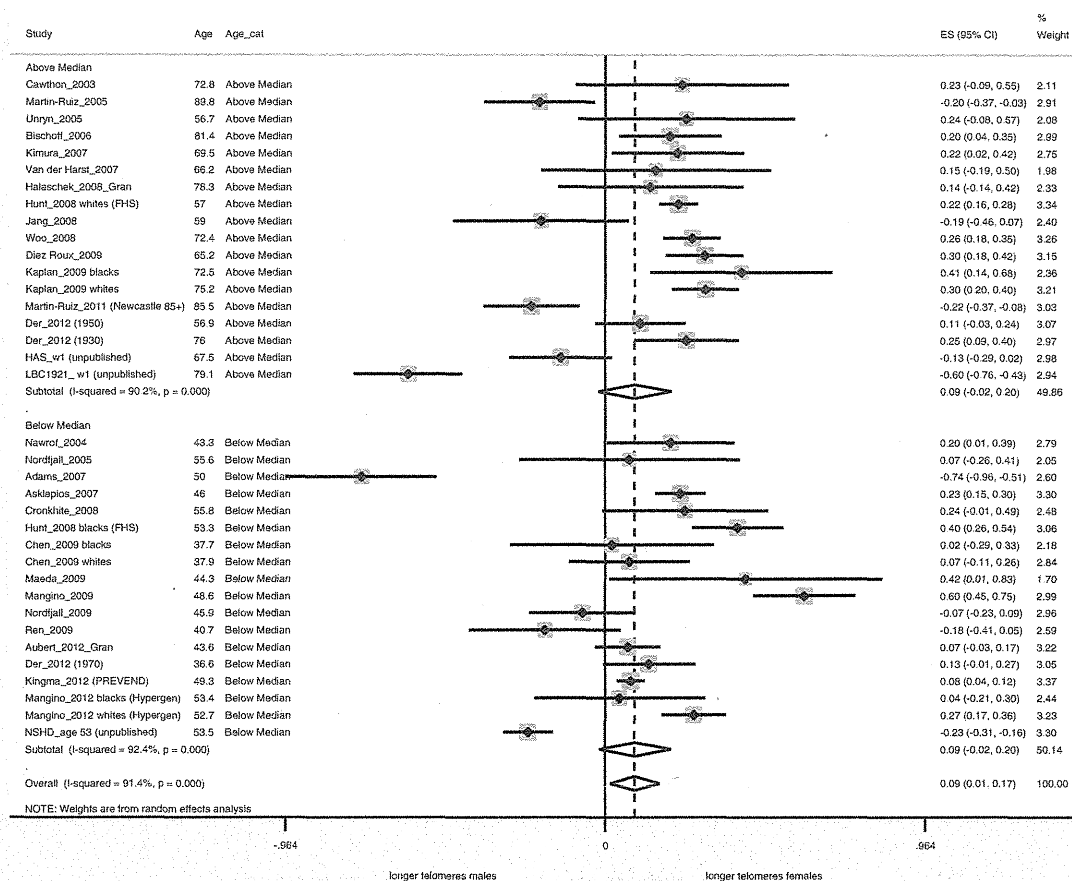


Fig. 3. Stratified meta-analyses above and below median age for the association between gender and telomere length adjusted for continuous age.

explanation for the fact that the Newcastle group found longer telomeres in men in most of their analysed cohorts, using Real-time PCR methodology.

Smaller measurement error in the TRF Southern Blot method as compared to the Real-time PCR methodology might explain greater consistency of findings between studies using the Southern blot technique (Aviv et al., 2011). In a recent blinded study (Aviv et al., 2011), the inter-assay CV was much larger (6.45%) for the Real-time PCR method performed by the Blackburn group than that of the TRF Southern Blot method as performed by the Aviv group (1.74%). However, this difference is probably not representative for the field as a whole. In the literature, inter-assay CV has ranged from 2.3% to 28% for Real-time PCR methods and from 1.5% to 12% for TRF Southern Blot methods (Aviv et al., 2011). In the studies included here, where reported, coefficient of variation ranged from 1.7% to 11.1% for Real-time PCR and from 1.4% to 12% for TRF Southern Blot. In a fully blinded comparative telomere length assessment involving 10 different groups, many of which also contributed data to the present analysis, median inter-assay CVs were not significantly different between techniques ($p = 0.86$, in preparation). Excluding the data with the lowest reported CVs generated by the Aviv group had little impact on effect size (0.174, 95% CI 0.045, 0.303, $p < 0.01$) or heterogeneity ($I^2 = 59.6\%$, $p < 0.03$) for the Southern blot results, although the average of the reported CVs for the remaining groups using the Southern blot technique was now 7.4%. Therefore, differences between Southern blotting and the two other techniques cannot be due to technology-specific random measurement error.

However, our results do not rule out the possibility of technology-specific bias. Various types of polymorphisms will have technology-specific impact on telomere length if measured by Southern blot versus PCR or Flow-FISH. The most probable of these are polymorphisms affecting the most distal recognition sites for the endonucleases Hinf1 and RSA1 typically used in TRF Southern blotting. Subtelomeric regions exhibit high levels of sequence polymorphism and strong linkage disequilibrium, leading to the existence of relatively few common haplotypes (Baird et al., 1995, 2000). The most distal Hinf1/RSA1 recognition site at chromosome X_p/Y_p, for instance, is polymorphic, resulting in an apparent increase in the length of this telomere if measured by Southern blotting in this haplotype (Baird et al., 1995). Interstitial telomere repeats are also highly polymorphic due to polymerase template slippage, which can cause either insertion or deletion of hexameric repeat units (Mondello et al., 2000). Both short (Ruiz-Herrera et al., 2008) and long interstitial telomeric repeats are frequent in the human genome, for instance an interstitial repeat at chromosome 22q11 displays length polymorphism ranging from 1 to 4 kb (Samassekou and Yan, 2011). Such interstitial sequences will either

Author's personal copy

Gender and telomere length stratified by measurement of telomere length

Study	Age	Measure	ES (95% CI)	% Weight
Real-time PCR				
Cawthon_2003	72.8	Real-time PCR	0.23 (-0.09, 0.55)	2.11
Martin-Ruiz_2005	89.8	Real-time PCR	-0.20 (-0.37, -0.03)	2.91
Nordfjall_2005	55.6	Real-time PCR	0.07 (-0.26, 0.41)	2.05
Adams_2007	50	Real-time PCR	-0.74 (-0.96, -0.51)	2.60
Van der Harst_2007	66.2	Real-time PCR	0.15 (-0.19, 0.50)	1.98
Jang_2008	59	Real-time PCR	-0.19 (-0.46, 0.07)	2.40
Woo_2008	72.4	Real-time PCR	0.26 (0.18, 0.35)	3.26
Diez Roux_2009	65.2	Real-time PCR	0.30 (0.18, 0.42)	3.15
Nordfjall_2009	45.9	Real-time PCR	-0.07 (-0.23, 0.09)	2.96
Martin-Ruiz_2011 (Newcastle 85+)	85.5	Real-time PCR	-0.22 (-0.37, -0.08)	3.03
Der_2012 (1970)	36.6	Real-time PCR	0.13 (-0.01, 0.27)	3.05
Der_2012 (1950)	56.9	Real-time PCR	0.11 (-0.03, 0.24)	3.07
Der_2012 (1930)	76	Real-time PCR	0.25 (0.09, 0.40)	2.97
Kingma_2012 (PREVEND)	49.3	Real-time PCR	0.08 (0.04, 0.12)	3.37
HAS_w1 (unpublished)	67.5	Real-time PCR	-0.13 (-0.29, 0.02)	2.98
LBC1921_ w1 (unpublished)	79.1	Real-time PCR	-0.60 (-0.76, -0.43)	2.94
NSHD_age 53 (unpublished)	53.5	Real-time PCR	-0.23 (-0.31, -0.16)	3.30
Subtotal (I-squared = 93.2%, p = 0.000)			-0.05 (-0.17, 0.07)	48.13
TRF Southern Blot				
Nawrot_2004	43.3	TRF Southern Blot	0.20 (0.01, 0.39)	2.79
Unryn_2005	56.7	TRF Southern Blot	0.24 (-0.08, 0.57)	2.08
Bischoff_2006	81.4	TRF Southern Blot	0.20 (0.04, 0.35)	2.99
Asklepios_2007	46	TRF Southern Blot	0.23 (0.15, 0.30)	3.30
Kimura_2007	69.5	TRF Southern Blot	0.22 (0.02, 0.42)	2.75
Cronkhite_2008	55.8	TRF Southern Blot	0.24 (-0.01, 0.49)	2.48
Hunt_2008 blacks (FHS)	53.3	TRF Southern Blot	0.40 (0.26, 0.54)	3.06
Hunt_2008 whites (FHS)	57	TRF Southern Blot	0.22 (0.16, 0.28)	3.34
Chen_2009 blacks	37.7	TRF Southern Blot	0.02 (-0.29, 0.33)	2.18
Chen_2009 whites	37.9	TRF Southern Blot	0.07 (-0.11, 0.26)	2.84
Kaplan_2009 blacks	72.5	TRF Southern Blot	0.41 (0.14, 0.68)	2.36
Kaplan_2009 whites	75.2	TRF Southern Blot	0.30 (0.20, 0.40)	3.21
Maeda_2009	44.3	TRF Southern Blot	0.42 (0.01, 0.83)	1.70
Mangino_2009	48.6	TRF Southern Blot	0.60 (0.45, 0.75)	2.99
Ren_2009	40.7	TRF Southern Blot	-0.18 (-0.41, 0.05)	2.59
Mangino_2012 blacks (Hypergen)	53.4	TRF Southern Blot	0.04 (-0.21, 0.30)	2.44
Mangino_2012 whites (Hypergen)	52.7	TRF Southern Blot	0.27 (0.17, 0.36)	3.23
Subtotal (I-squared = 68.8%, p = 0.000)			0.24 (0.17, 0.31)	46.32
Flow Fish				
Halaschek_2008_Gran	78.3	Flow Fish	0.14 (-0.14, 0.42)	2.33
Aubert_2012_Gran	43.6	Flow Fish	0.07 (-0.03, 0.17)	3.22
Subtotal (I-squared = 0.0%, p = 0.653)			0.08 (-0.02, 0.17)	5.55
Overall (I-squared = 91.4%, p = 0.000)			0.09 (0.01, 0.17)	100.00

NOTE: Weights are from random effects analysis

-.964 0 .964

longer telomeres males longer telomeres females

Standardised difference in telomere length between females and males adjusted for age

Fig. 4. Stratified meta-analyses by method of measurement of telomere length for the association between gender and telomere length adjusted for continuous age.

not contribute to the Southern blot signal at all (short interstitial sequences) or register as short telomere. In contrast, they will always increase the apparent average telomere length measured by PCR or Flow-FISH. In birds, the amount of interstitial telomeric sequence was substantial as compared to the actual telomeres and highly variable between species. Most importantly, it was also highly variable between individuals (Foote et al., 2013). It is not known whether frequencies of any of these polymorphisms might be gender-specific in humans. Finally, Hinf1/RSA1 are not fully methylation-insensitive, both may be blocked by overlapping CpG methylation. Telomeres tend to be transcriptionally silenced and hyper-methylated, however, this is a regulated process as evident in the human ICF syndrome, where hypomethylation due to lack of the DNA methyltransferase DNMT3b causes abnormally high levels of telomere repeat containing RNA (TERRA) and complete failure of telomere maintenance (Deng et al., 2010). Subtelomeric hypomethylation in endothelial cells was associated with apparent telomere lengthening as measured by Southern blot (Maeda et al., 2013). X inactivation in females might thus be expected to skew a subpopulation of telomeres towards apparently larger sizes if measured by Southern blot. Whether subtelomeric CpG sites on other chromosomes are differentially methylated between the sexes is not known but possible.

4.2. Strengths and limitations

This large scale systematic review and meta-analysis examining the association between gender and telomere length includes data from 40 datasets. We have followed a strict protocol, testing a priori hypotheses, have included several unpublished cohorts, have standardised regression coefficients and have undertaken a series of sensitivity analyses. We have thus aimed to minimise a range of biases including publication and selection bias. We requested that authors performed their analyses in a standard way, so limiting the heterogeneity caused by variation in analysis. Furthermore, possible sources of heterogeneity include the differences in the protocols for measuring telomere length, which might have biased the associations. We aimed to overcome such differences by standardising the regression coefficients and by running sensitivity analyses including raw telomere length data and transformed telomere length data ($\log_e$). We adjusted for age as a potential confounder. Other potential confounders include race, BMI and smoking status (Chen et al., 2011). Findings suggest leukocyte telomeres are longer in African Americans than in whites (Hunt et al., 2008), hence where available (Hunt et al., 2008; Chen et al., 2009; Kaplan et al., 2009) we stratified analyses by race. Shorter telomeres have been shown in some studies to be associated with obesity (Nordfjall et al., 2008b) and smoking Valdes et al., 2005). Only a small number of studies

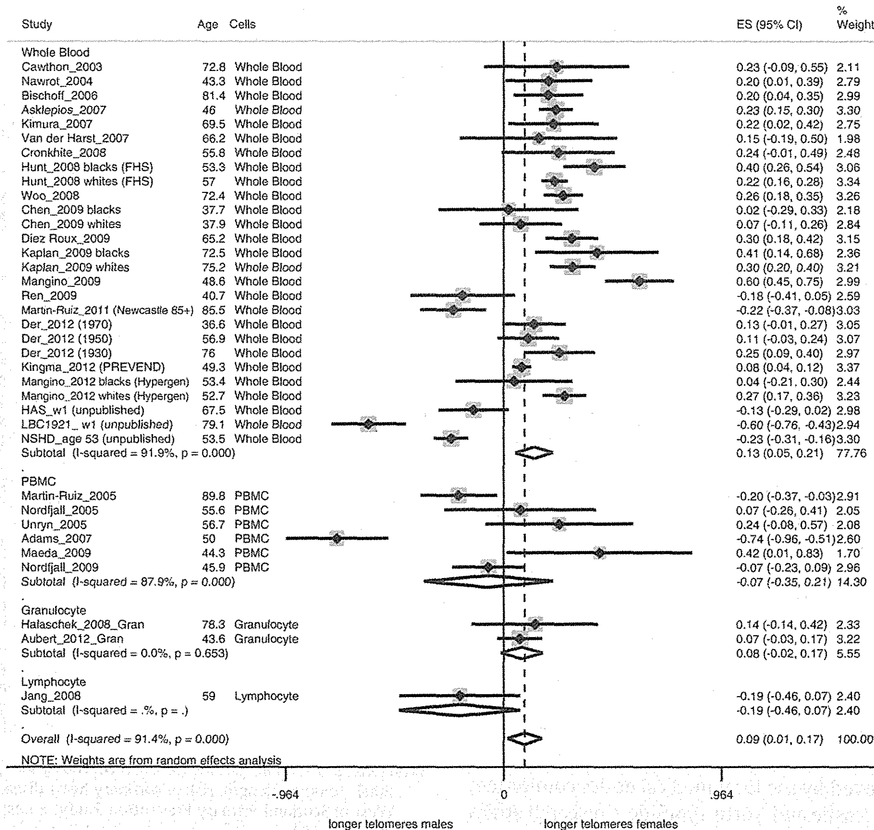


Fig. 5. Stratified meta-analyses by cell type for the association between gender and telomere length adjusted for continuous age.

included data on BMI and smoking status and we decided against requesting further adjustments for BMI and smoking status as this might lead to inconsistencies in adjustments. In addition to the characteristics investigated, it is possible that other factors might vary between studies, for example epigenetic status, proinflammatory state and pathogen challenge. All these factors interact with each other and with telomere length (Aviv et al., 2006; Bekaert et al., 2007; Shiels, 2010; Shiels et al., 2011) and could result in heterogeneity.

We also undertook several sensitivity analyses- including a control for measurement error by only including those studies with intra- or inter-assay CV measures and for four studies we included telomere length measures at phases 1 and 2 (up to 10 years apart) and we repeated the associations in separate meta-analyses at the two phases. For each of these sensitivity analyses, gender was still associated with telomere length, with females having longer telomeres than males. We did not assess the quality of the included studies, as quality assessment in meta-analysis of observational studies is controversial (Stroup et al., 2000). Although this was a large systematic review and meta-analysis, we might still have been underpowered for the sub-group analyses and meta-regression. Hence whilst the funnel plots and formal tests for publication bias gave no strong evidence for publication bias, these still need to be interpreted with caution. Mean ages in the study ranged from 36.6 ± 0.4SD years to 89.9 ± 3.2SD years, hence a limitation was that there were few studies at younger ages. We adjusted for age as a potential confounder but other potential confounders including BMI, smoking status and epigenetic status might have affected the strength of the associations. Furthermore, variation in these characteristics between the studies might result in heterogeneity.

5. Conclusion

Telomere length was longer on average in females than males and the strength of these associations varied by measurement method but not by age group: Southern blot was the only technique showing an unequivocal sex difference. This difference cannot be explained by differential random measurement error, but might be due to method-specific bias. Direct comparison of Southern blot results with at least one of the other methods in the same participants would help to clarify whether there are real sex differences in telomere length.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.exger.2013.12.004.

Funding

HALCyon is funded by the New Dynamics of Ageing (RES-353-25-0001) and MG and RC have received support from this grant. The NSHD is funded by the UK Medical Research Council (U120063239 and U123092720). TvZ acknowledges funding by MRC for telomere measurements (G0500997 and G0601333) and from BBSRC (BH092279) for a CASE studentship for LW. The Hertfordshire Ageing Study was funded

Author's personal copy

by the Wellcome Trust and the UK Medical Research Council. The Lothian Birth Cohort 1921 wave 1 phenotypic data collection and DNA preparation was funded by the Biotechnology and Biological Sciences Research Council (BBSRC) (project grant 15/SAG09977). The Lothian Birth Cohort 1921 wave 3 phenotypic data collection was funded by the Chief Scientific Office of the Scottish Government Health Directorates (project grant CZB/4/505). The LBC1921 work was undertaken by the University of Edinburgh Centre for Cognitive Ageing and Cognitive Epidemiology, part of the cross council Lifelong Health and Wellbeing Initiative (Centre grant G0700704/84698). Funding from the BBSRC, Engineering and Physical Sciences Research Council (EPSRC), Economic and Social Research Council (ESRC) and Medical Research Council (MRC) is gratefully acknowledged. The funders had no role in study design, data collection, analysis and interpretation of the data, decision to publish, or preparation of the manuscript.

Ethics statement

All included studies had received the relevant ethical approval. For the HALCyon cohorts: HAS: ethical approval was given by the Bedfordshire and Hertfordshire Local Research Ethics Committee and the West Hertfordshire Local Research Ethics Committee; LBC1921: Multicentre Research Ethics Committee for Scotland and the Lothian Research Ethics Committee; NSHD: North Thames Multi-Centre Research Ethics Committee (age 53y) and the Central Manchester Research Ethics Committee and the Scottish A Research Ethics Committee (age 60–64). Other relevant studies: HyperGEN: The Institutional Review Board of the University of Alabama at Birmingham Medical School; the Institutional Review Board of Boston University School of Medicine; the Institutional Review Board of the University of Minnesota Medical School; the Institutional Review Board of the University of Utah Medical School; The Institutional Review Board of the University of North Carolina; PREVEND: study was approved by the local medical ethics committee; Newcastle 85+ study: Newcastle and North Tyneside 1 research ethics committee; The West of Scotland Twenty-07 Study: Ethical approval for each wave was obtained from the relevant local ethics committee.

Conflict of interest disclosure

This is an original work, and it has never been published nor is under consideration for publication elsewhere. All the authors have substantially contributed to data collection and manuscript drafting and revision. Peter Lansdorp is a founding shareholder in Repeat Diagnostics, a company specializing in leukocyte telomere length measurements using flow FISH. All other authors have not declared any conflicts of interest.

Acknowledgements

We are very grateful to all cohort members who participated in the studies. Regarding the MESA study: This research was supported by contracts N01-HC-95159 through N01-HC-95169 from the National Heart, Lung, and Blood Institute and by grants UL1-RR-024156 and UL1-RR-025005 from NCRR. The authors thank the other investigators, the staff, and the participants of the MESA study for their valuable contributions. A full list of participating MESA investigators and institutions can be found at http://www.mesa-nhlbi.org. Regarding the Cardiovascular Health Study: This research was supported by grant HL80698 and contracts HHSN268201200036C, HHSN268200800007C, N01HC55222, N01HC85079, N01HC85080, N01HC85081, N01HC85082, N01HC85083, N01HC85086 and grant HL080295 from the National Heart, Lung and Blood Institute (NHLBI), with additional contribution from the National Institute of Neurological Disorders and Stroke (NINDS). Additional support from provided by AG023629 from the National Institute on Aging (NIA). A full list of principal CHS investigators and institutions can be found at http://www.chs-nhlbi.org/PI.htm. Jang et al.'s (2008) study was supported by a grant from the National R & D Program for Cancer Control, Ministry of Health & Welfare, Republic of Korea (0720550-2). We acknowledge the help of Christine Garcia, the corresponding author of the Cronkhite et al. (2008) study.

References

Adams, J., Martin-Ruiz, C., Pearce, M.S., White, M., Parker, L., von Zglinicki, T., 2007. No association between socio-economic status and white blood cell telomere length. Aging Cell 6, 125–128.
Aubert, G., Baerlocher, G.M., Vulto, I., Poon, S.S., Lansdorp, P.M., 2012. Collapse of telomere homeostasis in hematopoietic cells caused by heterozygous mutations in telomerase genes. PLoS Genet. 8. http://dx.doi.org/10.1371/journal.pgen.1002696.
Austad, S.N., 2006. Why women live longer than men: sex differences in longevity. Gend. Med. 3, 79–82.
Aviv, A., 2002. Telomeres, sex, reactive oxygen species, and human cardiovascular aging. J. Mol. Med. 80, 689–695.
Aviv, A., Valdes, A.M., Spector, T.D., 2006. Human telomere biology: pitfalls of moving from the laboratory to epidemiology. Int. J. Epidemiol. 35, 1424–1429.
Aviv, A., Hunt, S.C., Lin, J., Cao, X., Kimura, M., Blackburn, E., 2011. Impartial comparative analysis of measurement of leukocyte telomere length/DNA content by Southern blots and qPCR. Nucleic Acids Res. 39. http://dx.doi.org/10.1093/nar/gkr634.
Baird, D.M., Jeffreys, A.J., Royle, N.J., 1995. Mechanisms underlying telomere repeat turnover, revealed by hypervariable variant repeat distribution patterns in the human Xp/Yp telomere. EMBO J. 14, 5433–5443.
Baird, D.M., Coleman, J., Rosser, Z.H., Royle, N.J., 2000. High levels of sequence polymorphism and linkage disequilibrium at the telomere of 12q: implications for telomere biology and human evolution. Am. J. Hum. Genet. 66, 235–250.
Barrett, E.L., Richardson, D.S., 2011. Sex differences in telomeres and lifespan. Aging Cell 10, 913–921.
Bekaert, S., Rietzschel, E.R., De Buyzere, M.L., De Bacquer, D., Langlois, M., Segers, P., et al., 2007. Telomere length and cardiovascular risk factors in a middle-aged population free of overt cardiovascular disease. Aging Cell 6, 639–647.
Benetos, A., Okuda, K., Lajemi, M., Kimura, M., Thomas, F., Skurnick, J., et al., 2001. Telomere length as an indicator of biological aging: the gender effect and relation with pulse pressure and pulse wave velocity. Hypertension 37, 381–385.
Bischoff, C., Peterson, H.C., Graakjaer, J., Andersen-Ranberg, K., Vaupel, J.W., Bohr, V.A., et al., 2006. No association between telomere length and survival among the elderly and oldest old. Epidemiology 17, 190–194.
Brouilette, S.W., Moore, J.S., McMahon, A.D., Thompson, J.R., Ford, I., Shepherd, J., et al., 2007. Telomere length, risk of coronary heart disease, and statin treatment in the West of Scotland Primary Prevention Study: a nested case–control study. Lancet 369, 107–114.
Carrero, J.J., Stenvinkel, P., Fellstrom, B., Qureshi, A.R., Lamb, K., Heimburger, O., et al., 2008. Telomere attrition is associated with inflammation, low fetuin-A levels and high mortality in prevalent haemodialysis patients. J. Intern. Med. 263, 302–312.
Cawthon, R.M., Smith, K.R., O'Brien, E., Sivatchenko, A., Kerber, R.A., 2003. Association between telomere length in blood and mortality in people aged 60 years or older. Lancet 361, 393–395.
Chen, W., Gardner, J.P., Kimura, M., Brimacombe, M., Cao, X., Srinivasan, S.R., et al., 2009. Leukocyte telomere length is associated with HDL cholesterol levels: the Bogalusa heart study. Atherosclerosis 205, 620–625.
Chen, W., Kimura, M., Kim, S., Cao, X., Srinivasan, S.R., Berenson, G.S., et al., 2011. Longitudinal versus cross-sectional evaluations of leukocyte telomere length dynamics: age-dependent telomere shortening is the rule. J. Gerontol. A Biol. Sci. Med. Sci. 66, 312–319.
Cherkas, L.F., Hunkin, J.L., Kato, B.S., Richards, J.B., Gardner, J.P., Surdulescu, G.L., et al., 2008. The association between physical activity in leisure time and leukocyte telomere length. Arch. Intern. Med. 168, 154–158.
Collerton, J., Davies, K., Jagger, C., Kingston, A., Bond, J., Eccles, M.P., et al., 2009. Health and disease in 85 year olds: baseline findings from the Newcastle 85+ cohort study. BMJ 339. http://dx.doi.org/10.1136/bmj.b4904.
Costenbader, K.H., Prescott, J., Zee, R.Y., De Vivo, I., 2011. Immunosenescence and rheumatoid arthritis: does telomere shortening predict impending disease? Autoimmun. Rev. 10, 569–573.
Cronkhite, J.T., Xing, C., Raghu, G., Chin, K.M., Torres, F., Rosenblatt, R.L., 2008. Telomere shortening in familial and sporadic pulmonary fibrosis. Am. J. Respir. Crit. Care Med. 178, 729–737.
Deary, I.J., Whiteman, M.C., Starr, J.M., Whalley, L.J., Fox, H.C., 2004. The impact of childhood intelligence on later life: following up the Scottish mental surveys of 1932 and 1947. J. Pers. Soc. Psychol. 86, 130–147.
Deng, Z., Campbell, A.E., Lieberman, P.M., 2010. TERRA, CpG methylation and telomere heterochromatin: lessons from ICF syndrome cells. Cell Cycle 9, 69–74.
Der, G., Batty, G.D., Benzeval, M., Deary, I.J., Green, M.J., McGlynn, L., McIntyre, A., Robertson, T., Shiels, P.G., 2012. Is telomere length a biomarker for aging: cross-sectional evidence from the West of Scotland? PLoS One 7. http://dx.doi.org/10.1371/journal.pone.0045166.
DerSimonian, R., Laird, N., 1986. Meta-analysis in clinical trials. Control. Clin. Trials 7, 177–188.
Diez Roux, A.V., Ranjit, N., Jenny, N.S., Shea, S., Cushman, M., Fitzpatrick, A., et al., 2009. Race/ethnicity and telomere length in the multi-ethnic study of atherosclerosis. Aging Cell 8, 251–257.
Egger, M., Davey Smith, G., Schneider, M., Minder, C., 1997. Bias in meta-analysis detected by a simple, graphical test. BMJ 315, 629–634.

Fitzpatrick, A.L., Kronmal, R.A., Gardner, J.P., Psaty, B.M., Jenny, N.S., Tracy, R.P., et al., 2007. Leukocyte telomere length and cardiovascular disease in the cardiovascular health study. Am. J. Epidemiol. 165, 14–21.
Fitzpatrick, A.L., Kronmal, R.A., Kimura, M., Gardner, J.P., Psaty, B.M., Jenny, N.S., et al., 2011. Leukocyte telomere length and mortality in the cardiovascular health study. J. Gerontol. A Biol. Sci. Med. Sci. 66, 421–429.
Foote, C.G., Vleck, D., Vleck, C.M., 2013. Extent and variability of interstitial telomeric sequences and their effects on estimates of telomere length. Mol. Ecol. Resour. 13, 417–428.
Halaschek-Wiener, J., Vulto, I., Fornika, D., Collins, J., Connors, J.M., Le, N.D., et al., 2008. Reduced telomere length variation in healthy oldest old. Mech. Ageing Dev. 129, 638–641.
Harris, S.E., Deary, I.J., MacIntyre, A., Lamb, K.J., Radhakrishnan, K., Starr, J.M., et al., 2006. The association between telomere length, physical health, cognitive ageing, and mortality in non-demented older people. Neurosci. Lett. 406, 260–264.
Higgins, J.P., Thompson, S.G., 2002. Quantifying heterogeneity in a meta-analysis. Stat. Med. 21, 1539–1558.
Higgins, J.P., Thompson, S.G., Deeks, J.J., Altman, D.G., 2003. Measuring inconsistency in meta-analyses. BMJ 327, 557–560.
Horn, T., Robertson, B.C., Gemmell, N.J., 2010. The use of telomere length in ecology and evolutionary biology. Heredity 105, 497–506.
Hunt, S.C., Chen, W., Gardner, J.P., Kimura, M., Srinivasan, S.R., Eckfeldt, J.H., et al., 2008. Leukocyte telomeres are longer in African Americans than in whites: the National Heart, Lung, and Blood Institute Family Heart Study and the Bogalusa Heart Study. Aging Cell 7, 451–458.
Huzen, J., Peeters, W., de Boer, R.A., Moll, F.L., Wong, L.S., Codd, V., et al., 2011. Circulating leukocyte and carotid atherosclerotic plaque telomere length: interrelation, association with plaque characteristics, and restenosis after endarterectomy. Arterioscler. Thromb. Vasc. Biol. 31, 1219–1225.
Jang, J.S., Choi, Y.Y., Lee, W.K., Choi, J.E., Cha, S.I., Kim, Y.J., et al., 2008. Telomere length and the risk of lung cancer. Cancer Sci. 99, 1385–1389.
Kaplan, R.C., Fitzpatrick, A.L., Pollak, M.N., Gardner, J.P., Jenny, N.S., McGinn, A.P., et al., 2009. Insulin-like growth factors and leukocyte telomere length: the cardiovascular health study. J. Gerontol. A Biol. Sci. Med. Sci. 64, 1103–1106.
Kimura, M., Barbieri, M., Gardner, J.P., Skurnick, J., Cao, X., van Riel, N., et al., 2007. Leukocytes of exceptionally old persons display ultra-short telomeres. Am. J. Physiol. Regul. Integr. Comp. Physiol. 293, R2210–R2217.
Kuh, D., Pierce, M., Adams, J., Deanfield, J., Ekelund, U., Friberg, P., et al., 2011. Cohort profile: updating the cohort profile for the MRC National Survey of Health and Development: a new clinic-based data collection for ageing research. Int. J. Epidemiol. 40. http://dx.doi.org/10.1093/ije/dyq231.
Maeda, T., Guan, J.Z., Oyama, J., Higuchi, Y., Makino, N., 2009. Age-related changes in subtelomeric methylation in the normal Japanese population. J. Gerontol. A Biol. Sci. Med. Sci. 64, 426–434.
Maeda, T., Guan, J.Z., Koyanagi, M., Makino, N., 2013. Alterations in the telomere length distribution and the subtelomeric methylation status in human vascular endothelial cells under elevated temperature in culture condition. Aging Clin. Exp. Res. 25, 231–238.
Mangino, M., Richards, J.B., Soranzo, N., Zhai, G., Aviv, A., Valdes, A.M., et al., 2009. A genome-wide association study identifies a novel locus on chromosome 18q12.2 influencing white cell telomere length. J. Med. Genet. 46, 451–454.
Mangino, M., Hwang, S.J., Spector, T.D., Hunt, S.C., Kimura, M., Fitzpatrick, A.L., et al., 2012. Genome-wide meta-analysis points to CTC1 and ZNF676 as genes regulating telomere homeostasis in humans. Hum. Mol. Genet. 21. http://dx.doi.org/10.1093/hmg/dds382.
Martin-Ruiz, C.M., Gussekloo, J., van Heemst, D., von Zglinicki, T., Westendorp, R.G., 2005. Telomere length in white blood cells is not associated with morbidity or mortality in the oldest old: a population-based study. Aging Cell 4, 287–290.
Martin-Ruiz, C.M., Dickinson, H.O., Keys, B., Rowan, E., Kenny, R.A., von Zglinicki, T., 2006. Telomere length predicts poststroke mortality, dementia, and cognitive decline. Ann. Neurol. 60, 174–180.
Martin-Ruiz, C.M., Jagger, C., Kingston, A., Collerton, J., Catt, M., Davies, K., et al., 2011. Assessment of a large panel of candidate biomarkers of ageing in the Newcastle 85+ study. Mech. Ageing Dev. 132, 496–502.
Mayer, S., Bruderlein, S., Perner, S., Waibel, I., Holdenried, A., Ciloglu, N., et al., 2006. Sex-specific telomere length profiles and age-dependent erosion dynamics of individual chromosome arms in humans. Cytogenet. Genome Res. 112, 194–201.
Moher, D., Liberati, A., Tetzlaff, J., Altman, D.G., PRISMA Group, 2009. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. Ann. Intern. Med. 151, 264–269.
Mondello, C., Pirzio, L., Azzalin, C.M., Giulotto, E., 2000. Instability of interstitial telomeric sequences in the human genome. Genomics 68, 111–117.
Nawrot, T.S., Staessen, J.A., Gardner, J.P., Aviv, A., 2004. Telomere length and possible link to X chromosome. Lancet 363, 507–510.
Needham, B.L., Adler, N., Gregorich, S., Rehkopf, D., Lin, J., Blackburn, E.H., 2013. Socioeconomic status, health behaviour, and leukocyte telomere length in the National Health and Nutrition Examination Survey, 1999–2002. Soc. Sci. Med. 85. http://dx.doi.org/10.1016/j.socscimed.2013.02.023.
Nordfjall, K., Larefalk, A., Lindgren, P., Holmberg, D., Roos, G., 2005. Telomere length and heredity: indications of paternal inheritance. Proc. Natl. Acad. Sci. U. S. A. 102, 16374–16378.
Nordfjall, K., Eliasson, M., Stegmayr, B., Lundin, S., Roos, G., Nilsson, P.M., 2008a. Increased abdominal obesity, adverse psychosocial factors and shorter telomere length in subjects reporting early ageing; the MONICA Northern Sweden Study. Scand. J. Public Health 36, 744–752.
Nordfjall, K., Eliasson, M., Stegmayr, B., Melander, O., Nilsson, P., Roos, G., 2008b. Telomere length is associated with obesity parameters but with a gender difference. Obesity 16, 2682–2689.
Nordfjall, K., Svenson, U., Norrback, K.F., Adolfsson, R., Lenner, P., Roos, G., 2009. The individual blood cell telomere attrition rate is telomere length dependent. PLoS Genet. 5. http://dx.doi.org/10.1371/journal.pgen.1000375.
Okuda, K., Bardeguez, A., Gardner, J.P., Rodriguez, P., Ganesh, V., Kimura, M., et al., 2002. Telomere length in the newborn. Pediatr. Res. 52, 377–381.
Pellatt, A.J., Wolff, R.K., Torres-Mejia, G., John, E.M., Herrick, J.S., Lundgreen, A., et al., 2013. Telomere length, telomere-related genes, and breast cancer risk: the breast cancer health disparities study. Genes Chromosomes Cancer 52, 595–609.
Puterman, E., Epel, E.S., Lin, J., Blackburn, E.H., Gross, J.J., Whooley, M.A., et al., 2013. Multisystem resiliency moderates the major depression-telomere length association: findings from the Heart and Soul Study. Brain Behav. Immun. http://dx.doi.org/10.1016/j.bbi.2013.05.008.
Ren, F., Li, C., Xi, H., Wen, Y., Huang, K., 2009. Estimation of human age according to telomere shortening in peripheral blood leukocytes of Tibetan. Am. J. Forensic Med. Pathol. 30, 252–255.
Ruiz-Herrera, A., Nergadze, S.G., Santagostino, M., Giulotto, E., 2008. Telomeric repeats far from the ends: mechanisms of origin and role in evolution. Cytogenet. Genome Res. 122, 219–228.
Samassekou, O., Yan, J., 2011. Polymorphism in a human chromosome-specific interstitial telomere-like sequence at 22q11.2. Cytogenet. Genome Res. 134, 174–181.
Sanders, J.L., Fitzpatrick, A.L., Boudreau, R.M., Arnold, A.M., Aviv, A., Kimura, M., et al., 2012. Leukocyte telomere length is associated with noninvasively measured age-related disease: the cardiovascular health study. J. Gerontol. A Biol. Sci. Med. Sci. 67A, 409–416.
Shiels, P.G., 2010. Improving precision in investigating aging: why telomeres can cause problems. J. Gerontol. A Biol. Sci. Med. Sci. 65, 789–791.
Shiels, P.G., McGlynn, L.M., MacIntyre, A., Johnson, P.C.D., Batty, G.D., Burns, H., et al., 2011. Accelerated telomere attrition is associated with relative household income, diet and inflammation in the pSoBid cohort. PLoS ONE 6. http://dx.doi.org/10.1371/journal.pone.0022521.
Stroup, D.F., Berlin, J.A., Morton, S.C., Olkin, I., Williamson, G.D., Rennie, D., et al., 2000. Meta-analysis of observational studies in epidemiology: a proposal for reporting. Meta-analysis of Observational Studies in Epidemiology (MOOSE) group. JAMA 283, 2008–2012.
Syddall, H.E., Simmonds, S.J., Martin, H.J., Watson, C., Dennison, E.M., Cooper, C., et al., 2010. Cohort profile: the Hertfordshire Ageing Study (HAS). Int. J. Epidemiol. 39, 36–43.
Thompson, S.G., Sharp, S.J., 1999. Explaining heterogeneity in meta-analysis: a comparison of methods. Stat. Med. 18, 2693–2708.
Unryn, B.M., Cook, L.S., Riabowol, K.T., 2005. Paternal age is positively linked to telomere length of children. Aging Cell 4, 97–101.
Valdes, A.M., Andrew, T., Gardner, J.P., Kimura, M., Oelsner, E., Cherkas, L.F., et al., 2005. This title is Obesity, cigarette smoking, and telomere length in women. Lancet 366, 662–664.
van der Harst, P., van der Steege, G., de Boer, R.A., Voors, A.A., Hall, A.S., Mulder, M.J., et al., 2007. Telomere length of circulating leukocytes is decreased in patients with chronic heart failure. J. Am. Coll. Cardiol. 49, 1459–1464.
von Zglinicki, T., 2002. Oxidative stress shortens telomeres. Trends Biochem. Sci. 27, 339–344.
von Zglinicki, T., 2012. Will your telomeres tell your future? BMJ 344. http://dx.doi.org/10.1136/bmj.e1727.
von Zglinicki, T., Serra, V., Lorenz, M., Saretzki, G., Lenzen-Grossimlighaus, R., Gessner, R., et al., 2000. Short telomeres in patients with vascular dementia: an indicator of low antioxidative capacity and a possible risk factor? Lab. Invest. 80, 1739–1747.
Weischer, M., Bojesen, S.E., Cawthon, R.M., Freiberg, J.J., Tybjaerg-Hansen, A., Nordestgaard, B.G., 2012. Short telomere length, myocardial infarction, ischemic heart disease, and early death. Arterioscler. Thromb. Vasc. Biol. 32, 822–829.
Weischer, M., Nordestgaard, B.G., Cawthon, R.M., Freiberg, J.J., Tybjaerg-Hansen, A., Bojesen, S.E., 2013. Short telomere length, cancer survival, and cancer risk in 47102 individuals. J. Natl. Cancer Inst. 105, 459–468.
Woo, J., Tang, N.L., Suen, E., Leung, J.C., Leung, P.C., 2008. Telomeres and frailty. Mech. Ageing Dev. 129, 642–648.
Yamaguchi, H., Calado, R.T., Ly, H., Kajigaya, S., Baerlocher, G.M., Chanock, S.J., et al., 2005. Mutations in TERT, the gene for telomerase reverse transcriptase, in aplastic anemia. N. Engl. J. Med. 352, 1413–1424.

Manuscript Num: ACB_1602
Journal Name : Applied Cell Biology
Manuscript Title : Vascular endothelial cell surviving through under prolonged elevated temperature shows persistent or transient up-regulation of telomerase and stress-associated proteins.
Article Type : Full Paper
Corresponding Author : Toyoki
Corresponding Author Affiliation : Kyushu University
Corresponding Author Email-Address : maedat@beppu.kyushu-u.ac.jp

Regular Paper

Vascular endothelial cell surviving through under prolonged elevated temperature shows persistent or transient up-regulation of telomerase and stress-associated proteins

Toyoki Maeda[1]*, Guan Jing-Zhi[2], Masamichi Koyanagi[1], Naoki Makino[1]
[1]The Department of Cardiovascular, Respiratory and Geriatric Disease, Kyushu University Beppu Hospital, Beppu, Oita, 874-0838, (JAPAN)
[2]The 309th Hospital of Chinese People's Liberation Army, Beijing, (CHINA)
E-mail : maedat@beppu.kyushu-u.ac.jp

ABSTRACT

The effects of heat on vascular endothelial cells are studied. Telomere of human umbilical venous endothelial cells (HUVECs) cultured at 42°C was studied. The expression of several factors concerning to telomere maintenance and vascular endothelial physiology was analyzed.
HUVECs were cultured for 1 day or for 3 days under 42°C. Their telomere lengths and telomere length distributions were analyzed and compared with those at 37°C. The telomerase activity and the expression of telomere-associated RNA, telomere-associated proteins (TERC, TERT, TRF1, and TRF2), heat shock proteins (Hsp60, Hsp70, and Hsp90) and endothelial nitric oxide synthase were also analyzed.
The cell growth was suppressed both on day1 and on day3 at 42°C. Unexpectedly, however, the cell senescence rate was elevated only on day1 at 42°C, compared to that at 37°C. The mean telomere length was not different between at 37°C and at 42°C, whereas in the telomere length distribution long telomere decreased and middle-sized telomere increased only on day1 at 42°C. Telomere-associated proteins, heat shock proteins, and NOS were up-regulated at day1, and the up-regulation disappeared at day3.
The results suggested that long telomeres were affected and various genes are up-regulated in reaction to elevated temperature, and that the cells surviving through the prolonged exposure to heat lose the early responses. The cell subpopulation bearing long telomere seemed more sensitive to heat stress. The observed initial up-regulation of telomere-associated proteins and others showed an aspect of heat responses of vascular endothelial cells. Among the responses, some seemed to be favor for the biological function and the survival of vascular endothelial cell, for example, the upregulation of telomerase activity and the elevated expression of TERT, heat shock protein, and NOS. In addition, an upregulation was maintained uniquely in Hsp70 at a later stage of heat exposure. This observation implicates that the upregulation of Hsp70 play an important roll for cell survival under prolonged heat stress.
© 2014 Trade Science Inc. - INDIA

KEYWORDS

Vascular endothelial cell;
Telomere;
Heat shock protein.

INTRODUCTION

Telomere consists of repetitive DNA sequence with accessory protein components (TRF1, TRF2, and others) capping the terminals of chromosome[1]. It is well known that telomere DNA shortening proceeds at every cell cycle due to a duplication process yielding a little shorter DNA strand. The telomere length of somatic cells is negatively affected by stress factors including oxidative stress[2]. Pathological mental and physical stress accelerates telomere attrition[2]. Telomere shortening is considered to occur in somatic cells with aging through many rounds of cell cycle and pathological stress[3]. On the other hand, there is a telomere-elongating cellular mechanism, which functions in limited cases. Telomerase consists of a protein component of reverse transcriptase (TERT) and a RNA part (TERC). Telomerase contributes the telomere elongation or telomere length maintenance in unique cell population with active mitotic potential, such as cancer cells, stem cells, and reproductive cells. Generally, the telomere activity is, however, suppressed to a low level in the somatic cells, which is not enough to prevent the telomere shortening with cell divisions. Telomerase activity is also affected by stress factors. However, the effect of heat stress on telomere length or telomerase has not been well studied. Heat shock proteins (Hsp) are ubiquitously synthesized in virtually all species and it is hypothesized that they might have beneficial health effects[4]. In response to stress stimuli, mammalian cells activate a signaling pathway leading to the transient expression of heat shock proteins (Hsp). Hsp's are a family of proteins serving as molecular chaperones that prevent the formation of nonspecific protein aggregates and assist proteins in the acquisition of their native structures. Physiologically, Hsp's play a protective role in the homeostasis of the vessel wall consisting of endothelial cells and smooth muscle cells[5]. Previous reports have shown that heat-associated vasodilatation is associated with vascular NO synthesis[6]. Vascular endothelial cells express endothelial nitric oxide synthase (eNOS). eNOS mediates NO synthesis in vascular endothelial cells, which relaxes the surrounding smooth muscle cells of vascular wall to vasodilatation. NO synthesis has been regarded as a functional marker for vascular endothelial cells. We pursued the heat-associated alteration in the vascular reactivity for vasodilatation of cultured HUVECs by assessing the eNOS expression level of HUVECs under heat-exposure. Heat-associated effects on genomic DNA including telomere erosion, have not been well investigated. Here we analyzed telomere DNA, and the expressions of telomere-associated components, heat shock proteins and nitric oxide synthase in reaction to heat-stress by using vascular endothelial cells in culture exposed to heat.

MATERIALS AND METHODS

Cell culture

Human umbilical venous endothelial cells (HUVEC) were purchased from Clonetics Corp. (San Diego, CA). The cells were cultured in endothelial cell growth medium (Clonetics Corp.) at 37°C or 42°C and 5% CO_2 in a gelatin-coated flask (Iwaki Glass, 2 Chiba, Japan). Culture media were refreshed every 24 h. On day 1 or 3, the cells were collected and were counted using a hemocytometer. Population doublings (PDs) were calculated using the formula: PD=[log (expansion)/log2], where expansion was the number of cells harvested divided by the initial number of cells seeded.

Senescence-associated β-galactosidase (SA-β-Gal) expression

The cells were washed in PBS, fixed for 10 min at room temperature in 2% formaldehyde/0.2% glutaraldehyde, and incubated at 37°C (no CO_2) with fresh SA-β-Gal staining solution containing 1mg/mL of X-gal, pH 6.0 for 12h. One hundred cells were scored from each well (plate) using a light microscope.

Telomere detection

Telomere detection was performed as previously described[7]. Blood cell DNA was extracted from samples and the DNA (0.1ug) were digested with *Msp*I. The digests (10ul) were subjected to Southern blot hybridization with a telomere DNA probe. The autoradiogram was captured on an Image Master, and the telomere length was then assessed quantitatively. Every sample was measured in triplicate.

Terminal length (TL) analysis

Telomere length distribution was analyzed by com-

paring the telomere length using a telomere percentage analysis with three intervals of length (>9.4, 9.4≥≥4.4 and <4.4kb) as defined by a molecular weight standard as previously described[7]. The percent of the stratified intensity in each molecular weight range was measured. The mean TL was estimated using the formula S(ODi - background)/ S(ODi – background /Li), where ODi is the chemiluminescent signal and Li is the length of the TRF fragment at position i.

Semiquntitative RT-PCR for TERC RNA

Total RNA samples were extracted using RNAzol B (Teltest). mRNA for human telomerase RNA component (TERC) was determined by RT-PCR using a DIG detection system (Roche Applied Science). Each human cDNA was produced by RT-PCR according to each human-derived sequence. For the amplification of β-actin cDNA, the forward primer β-actin (205bp) 5' -CCTTCCTGGGCATGGAGTCCT-3' and the reverse primer 5'-GGAGCAATGATCTTGATCTTC-3' were used according to the published human TERC cDNA sequence[8]. And TERC forward primer 5'-TCTAACCCTAACTGAGAAGGGCGTAG-3' reverse primer 5'-GTTTGCTCTAG AATGAACGGTGGAAG-3' were used[9]. The values for TERC mRNA levels were normalized to the β-actin mRNA level in the same sample. The PCR products were directly synthesized from 2 μg of total RNA isolated from each sample using the Superscript one-step RT-PCR system with Platinum *Taq* (Invitrogen) and gene-specific primers according to the recommendations provided by the supplier. The PCR products were analyzed by agarose gel electrophoresis (1.3%) followed by staining with ethidium bromide and scanning with Gel-Doc (Bio-Rad). For semiquantitative PCR, β-actin was used as an internal control to evaluate total RNA input, as described by our group[10].

Western blot and other analyses

Cells from a dish were homogenized with 100μl lysis buffer (100mM Tris pH 6.8, 4% SDS, 20% glycerol containing the protease inhibitor, M phenylmethanesulfonyl fluoride, 0.1mM, leupeptin, 0.1μl, and aprotinin, 0.1μl). Gel electrophoresis was used to separate 10μg protein on a 10% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membranes (162-0112, Bio-Rad Labo-ratories, Hercules, California) blocked with 5% dry milk or blocking solution for Western blot (Roche). Membranes were blocked and incubated with antibodies against telomerase reverse transcriptase (TERT) (Rockland), TRF1 (Imgenex), TRF2 (Cell Signaling), Hsp60 (Assay designs), Hsp70 (Assay designs), Hsp90 (Enzo), eNOS (BD Biosciences), phospho-eNOS (Cell Signaling), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology). Detection was performed with secondary horseradish peroxidase-conjugated antibodies (Chemicon) and the ECL detection system as previously described[11]. The relative expression levels were determined compared to that of GAPDH.

Telomerase activity

Telomerase activity was examined by means of a modified telomerase repeat amplification protocol (TRAP) method with TeloChaser (Toyobo, Osaka, Japan), as previously described[12].

Statistical analysis

Assays were repeated three times and analyzed statistically. The normality of the data was examined with the Kolmogorov–Smirnov test and the homogeneity of variance with the Levene Median test. If both the normal distribution and equal variance tests were passed, intergroup comparisons were performed using a two-way analysis of variance (ANOVA) test followed by all pairwise multiple comparison procedures using Tukey's post hoc test. The data are expressed as the mean±standard deviation. The criterion for the significance is $p<0.05$.

RESULTS

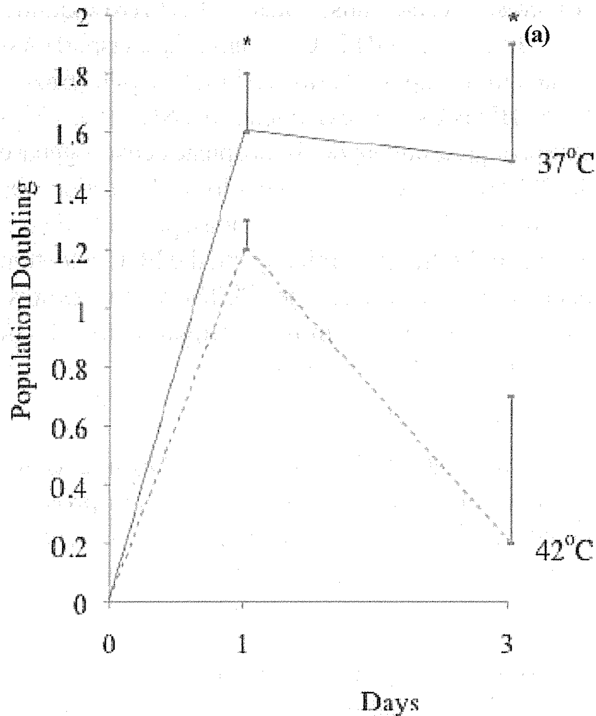
Population doubling (PD) and Cell senescence

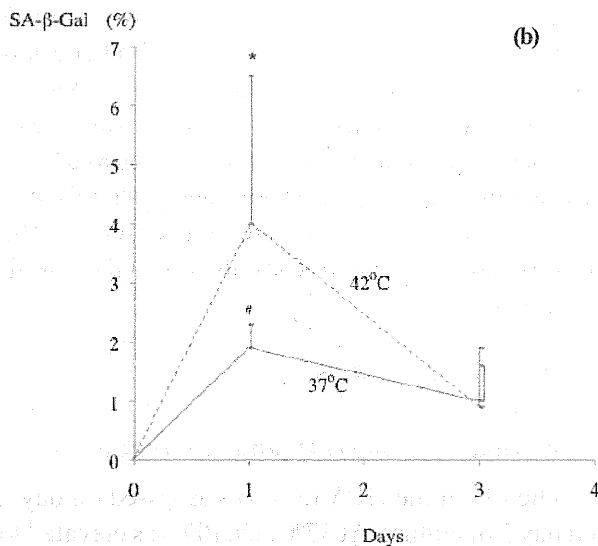
The PD of the HUVECs was assessed on day 1 and day 3 of culture. At 37°C, the PD was elevated to ~1.6 on day1 and the PD value was maintained on day3. At 42°C, initially the PD was elevated to ~1.2, but decreased steeply to ~0.2 on day3 (Figure 1a).

Senescence-associated β-galactosidase (SA-β-Gal) expression was observed in ~2% of cells on day1 and ~1% on day3 at 37°C, and ~4% on day1 and

~2% on day3 at 42°C (Figure 1b).

The PD value did not increased at 37°C, and decreased at 42°C during day1 and day3. This apparent suppress of cell growth might be derived partly from damaged subpopulation during cell preparation before starting culture being left. The elevated cell senescence on day1 both at 37°C and at 42°C supported this idea. The difference between PD and cell senescence were derived from heat stress, and cells were suppressed in growth potential at 42°C. Cell population survived through the heat condition of 42°C for 3 days bore a similar proportion of senescent cells as at 37°C. This indicated that heat-sensible cells were completely eradicated during day1 and day3 at 42°C. As a result, heat-durable cells survived on day3 at 42°C.





a. The PD on day1 and day3 at 37°C and 42°C. The horizontal bars are standard deviations. *p<0.05, at 37°C vs at 42°C on day1. b. The percentages of SA-b-Gal-positive cells. *p<0.05, at 37°C vs at 42°C. #p<0.05, on day1 vs on day3.

Figure 1 : The population doubling (PD) and the ratio of senescence-associated b-galactosidase (SA-β-Gal) staining of HUVECs cultured in the presence of H_2O_2

The mean TRF level and its distribution

The mean TL's of HUVECs were measured to assess how much high temperature condition affected the telomeric DNA (TABLE 1). At 42°C, long telomere (>9.4kb) decreased on day1 and middle-sized telomere (9.4kb-4.4kb) increased. These differences between 37°C and 42°C disappeared on day3. The proportions between values at 37°C and those at 42°C were also compared to pursue the heat-specific changes from day1 to day3 (TABLE 2). The decrease of long telomere and the increase of middle-sized telomere were observed only on day1 and the short telomere (<4.4kb) was not significantly affected. These changes of TL distribution disappeared on day3. Unexpectedly, cells with longer telomere seemed more easily affected by heat stress, compared to those with shorter telomere.

TABLE 1 : The telomere length and the telomere length distribution of HUVECs exposed to heat

	37°C		42°C	
	Day1	Day3	Day1	Day3
MspI-TL(kb)	9.2±1.0	8.7±0.3	8.3±1.0	8.9±0.6
>9.4kb(%)	51±8	46±1	39±11*	49±5
9.4-4.4kb(%)	47±6	51±2	59±10*	48±3
<4.4kb(%)	2±3	3±2	2±1	4±2

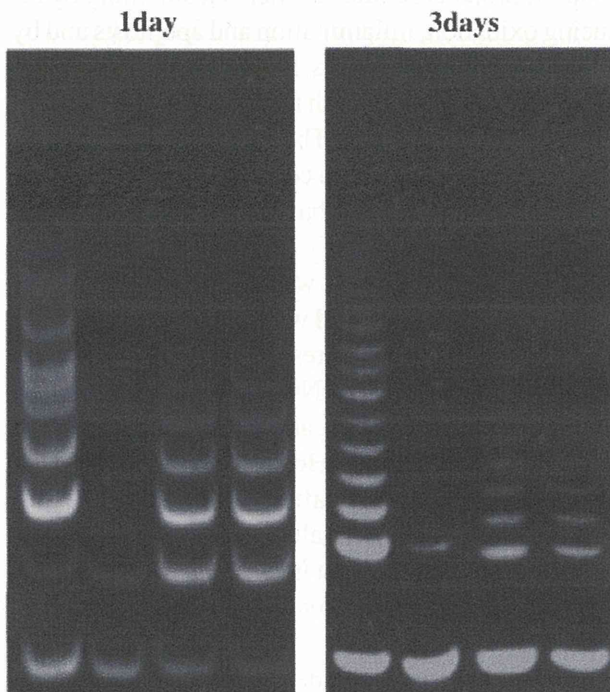
*37°C vs42°C p<0.05

TABLE 2 : The proportional values of telomere length parameters at 42°C compared to those at 37°C

	Day1	Day3	p-value
MspI-TL	0.90±0.11	1.02±0.07	0.06
>9.4kb	0.76±0.21	1.06±0.10	0.01
9.4-4.4kb	1.26±0.20	0.93±0.06	0.01
<4.4kb	1.01+±0.74	1.21±0.69	0.63

Telomerase activity

The telomerase activity of HUVECs at 37°C and 42°C was evaluated by TRAP assay (Figure 2). Average value of TPG at 37°C was put as 1(1±0.66 for 1day, 1±0.2 for 3days). The relative TPG7s at 42°C were 1.32±0.84 on day1 and 0.57±0.28 on day3. Thus, the relative telomerase activity of HUVECs at 42°C was maintained on day1 (p= 0.71) but significantly decreased on day3 (p=0.03). Telomerase activity decreased under prolonged heat stress at 42°C.



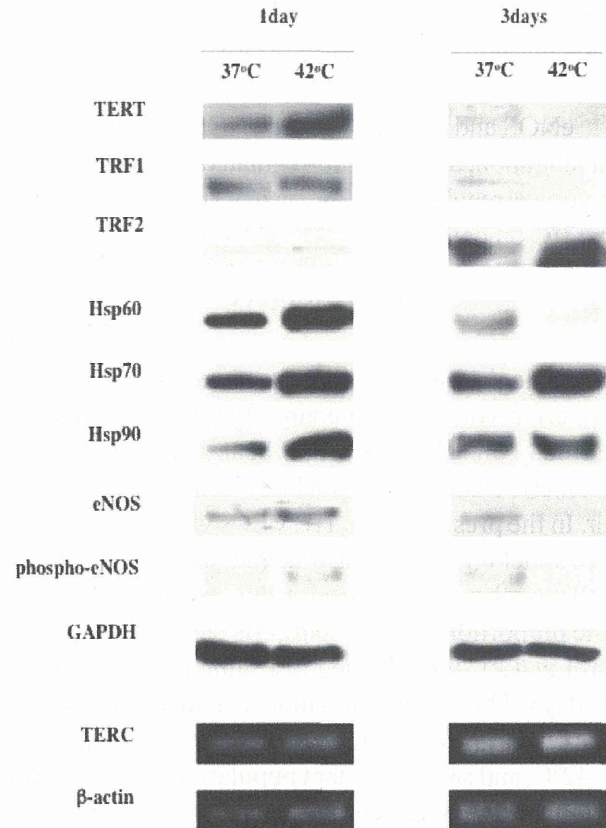
The photographs of representative TRAP assay results are shown. The materials used for the positive control (pc) and negative control (nc) were provided with the kit

Figure 2 : The telomerase activity of endothelial cells in the presence of H_2O_2

Expression of telomere-associated components and others

All the three telomere-associated components (TERT, TRF1, and TRF2) except TERC were up-regulated on day1, thereafter were down-regulated on day3. TERC did not seem to be affected by heat in the expression. (Figure 3, TABLE 3).

All the analyzed heat shock proteins (Hsp60, hsp70, and hsp90) were up-regulated at 42°C on day1 and



The representative Western blot results of telomere-associated proteins (TERT, TRF1, and TRF2), heat shock proteins (Hsp60, Hsp70, and Hsp90) and a TERC RNA RT-PCR result are shown. The relative expression level of each component is shown in TABLE 3

Figure 3 : The expression levels of TERC, telomere-associated proteins, heat shock proteins, and NOS of HUVECs cultured on day1 and day3 at different temperatures

TABLE 3 : The proportional values of the expressions of the telomere-associated components, heat shock proteins and eNOS at 42°C compared to those at 37°C

	Day1	Day3	p-value
TERT	1.80±0.32	0.25±0.14	0.01>
TRF1	1.43±0.19	0.44±0.25	0.01>
TRF2	3.93±1.45	1.19±1.07	0.06
Hsp60	2.35±0.58	0.17±0.11	0.02
Hsp70	2.73±0.08	1.46±0.08	0.00
Hsp90	3.35±1.43	0.89±1.46	0.08
eNOS	2.43±0.52	0.25±0.04	0.02
TERC	0.95±0.27	1.86±1.66	0.44

The photo results are shown in Figure 3. The relative expression levels were determined by the proportion of each band density to that of β-actin or GAPDH (set at 1 each), β-actin (RNA) for TERC and GAPDH (protein) for western results

down-regulated day3. However, only hsp70 still kept a significantly higher expression level at 42°C compared to at 37°C.

eNOS and phosphorylated eNOS showed a similar alteration, they were up-regulated at 42°C on day1, but down-regulated at 42°C on day3 to below the level at 37°C on day3.

DISCUSSION

Biological stress has been reported to induce negative effects on the maintenance of telomere length in various cells[1,13]. However, heat stress-associated telomeric changes have not been well investigated, so far. In the present study, HUVECs were used to analyze the telomere-associated alterations induced at 42°C. Cell growth was suppressed at 42°C especially on day3. The proportion of senescent cells elevated indeed on day1 at 42°C but returned to a same level as at 37°C on day3. This observation indicated that a heat-sensitive cell population diminished during 3-day-exposure to 42°C, and a heat-resistant population survived until day3. In the telomere length distribution, apparently long telomere decreased and telomere of medium size (4.4-9.4kb) increased at 42°C on day1. This indicated that growth rate was suppressed more in cells with long telomere than those with short telomere under exposure to heat-stress in an early stage of heat exposure. The shortest range of telomere (<4.4kb) showed an increasing trend of increase at 42°C on day3, although it was not significant. A part of cell population bearing short telomeres might be damaged and diminished at 42°C, resulting in non-clear increase of them on day3. The grown subpopulation at 42°C by day1 seemed to diminish by day3, because the TL distribution of the survived cell population at 42°C on day3 returned to a similar pattern at 37°C on day3.

This suggested that a subpopulation of cells with short telomere grew initially in heat-exposure but lost the heat-endurance during a prolonged heat exposure. The mechanism of the apparent heat-associated growth suppression of cells with long telomere is not clear, so far. Cells containing long telomeres, i.e., young cells, may be equipped with an unknown mechanism suppressing cell growth under a heat-stressed condition, possibly being a favor for the cell survival.

All the analyzed proteins up-regulated on day1 at 42°C. The survived cells for 3 days at 42°C revealed an elevated expression of Hsp70, and maintained the expression of TERC, TRF2, and Hsp90. All the others, TERT, TRF1, Hsp60, and NOS were suppressed in expression at 42°C. The expression of Hsp70 might contribute to survival advantage under a prolonged heat-exposure. We here show that an up-regulation after 3-day-culture at 42°C is maintained only in Hsp70 among analyzed heat shock proteins. Heat shock protein70 (Hsp70) protects cellular elements from injury by reducing oxidation, inflammation and apoptosis and by refolding damaged proteins. Hsp70 improves viability of stressed vascular smooth muscle cells, possibly via its chaperone functions[14]. The beneficial effects on cell viability are here shown to contribute not only to vascular smooth muscle cells but also possibly to vascular endothelial cells.

The expression of NOS was up-regulated in an early stage of heat-exposure and was down-regulated later. This suggested that heat-stress had an inducible potential for NOS expression. NOS has been reported to contribute to vasodilatation, and so heat exposure would result in vasodilatation[6]. However the vasodilatation by NOS would diminish after prolonged heat exposure. The heat-associated alterations of NOS expression seemed rather to result in changing vasodilatation potential than contribute to survival advantage for heat stress.

Longer telomeres seemed transiently affected under exposure to heat stress. However, prolonged heat exposure did not leave a persistent altered pattern of telomere length distribution. In contrast to the growth suppression and the down-regulation of various proteins including telomere-associated proteins, other heat shock proteins, and NOS, only Hsp70 up-regulated persistently under heat stress. Hsp70 may be a strong survival factor for heat-damaged cells. Further study is necessary to confirm the vascular cellular heat-resistance associated with Hsp70.

ACKNOWLEDGMENT

We would like to thank Ms. K. Tsuchida, Ms. S. Taguchi, and Ms. Y. Ueda for their expert technical assistance. This work was supported by grants from the

Regular Paper

Ministry of Education, Science, and Culture of Japan (#23590885), the National Natural Science Fund (NSFC) (81170329/H2501), and 2012 Health and Labour Sciences Research Grants Comprehensive Research on Life Style Related Diseases including Cardiovascular Diseases and Diabetes Mellitus.

REFERENCE

[1] E.H.Blackburn; Nature (London), **350**, 569-573 **(1991)**.

[2] T.Maeda, J-Z.Guan, J.Oyama, Y.Higuchi, N.Makino: Journal of Gerontology: Series A Biological Sciences and Medical Sciences, **64**, 949-955 **(2009)**.

[3] G.Aubert, P.M.Lansdorp; Physiological Review, **88**, 557-579 **(2008)**.

[4] R.Njemini, I.Bautmans, O.O.Onyema, K.Van Puyvelde, C.Demanet, T: BMC Immunology, **12**, 24 **(2011)**.

[5] Q.Xu, B.Metzler, M.Jahangiri, K.Mandal: American Journal of Physiology Heart and Circulation Physiology, **302**, H506-514 **(2012)**.

[6] D.L.KJr.ellogg, C.G.Crandall, Y.Liu, N.Charkoudian, J.M.Johnson: Journal of Applied Physiolology, **85**, 824-829 **(1998)**.

[7] J-Z.Guan, T.Maeda, M.Sugano, J.Oyama, Y.Higuchi, N.Makino; Molecular Cellular Biochemistry, **304**, 253–260 **(2007)**.

[8] Q.Tao, B.Lv, B.Qiao, C.Q.Zheng, Z.F.Chen; Oral Oncology, **45**, e239-244 **(2009)**.

[9] M.Fujiwara, H.Kamma, W.Wu, Y.Yano, S.Homma, H.Satoh; International Journal of Oncology, **29**, 445-451 **(2006)**.

[10] N.Makino, T.Maeda, J-I.Oyama, Y.Higuchi, K.Mimori: American Journal of Physiology Heart and Circulation Physiology, **297**, H2188–195 **(2009)**.

[11] M.Sugano, K.Tsuchida, T.Maeda, N.Makino; Atherosclerosis, **191**, 33–39 **(2007)**.

[12] T.Maeda, R.Kurita, T.Yokoo, K.Tani K, N.Makino: Biochemical and Biophysical Research Communications, **407**, 491-494 **(2011)**.

[13] V.A.Zakian; Science, **270**, 1601-1607 **(1995)**.

[14] A.Bielecka-Dabrowa, M.Barylski, D.P.Mikhailidis, J.Rysz, M.Banach: Expert Opinion on Therapeutic Targets, **13**, 307-317 **(2009)**.

Manuscript Num: ACB_1592
Journal Name : Applied Cell Biology
Manuscript Title : X-irradiation alters the telomerase activity and the telomere length distribution of cultured human vascular endothelial cells
Article Type : Full Paper
Corresponding Author : Toyoki
Corresponding Author Affiliation : Kyushu University
Corresponding Author Email-Address : maedat@beppu.kyushu-u.ac.jp

Regular Paper

X-irradiation alters the telomerase activity and the telomere length distribution of cultured human vascular endothelial cells

Toyoki Maeda[1]*, Jing-Zhi Guan[2], Masamichi Koyanagi[1], Naoki Makino[1]
[1]The Department of Cardiovascular, Respiratory, and Geriatric Medicine, Kyushu University Beppu Hospital, Beppu, Oita, 874-0838, (JAPAN)
[2] The 309th Hospital of Chinese People's Liberation Army, Beijing, (CHINA)
E-mail : maedat@beppu.kyushu-u.ac.jp

ABSTRACT

To assess the effect of X-irradiation on somatic cell, we analyzed the telomeric changes of cultured human umbilical venous endothelial cells (HUVECs) induced shortly after low-dose X-irradiation. The effect of X-irradiation on HUVECs was assessed by the analysis of the changes in telomere length, telomerase activity, and the expression of telomere-associated proteins after 2 to 8Gy X-irradiation. The cell growth activity decreased, whereas the telomerase activity of the surviving cells decreased only at low X-ray doses. The expression levels of telomere-associated components, TRF1 and TRF2, increased in the surviving cells. As the X-ray dose level increased, senescent cells increased. However, the mean telomere length of the surviving cells became longer, long telomeres increased, and short telomeres decreased. These observations suggested that X-irradiated HUVECs bore telomeric features similar to those of young cells, and the cells bearing short telomeres, i.e., aged cells, were selected out. The surviving cells that had gone through low dose X-irradiation might represent a radiation-resistant feature of telomere conditions. The telomeric changes at low dose X-ray disappeared at a high dose. Higher dose of X-irradiation might induce a cellular protective reaction against X-ray-induced cell damage through the restoration of telomerase activity and up-regulated telomere-associated proteins.
© 2014 Trade Science Inc. - INDIA

KEYWORDS

Telomere;
Telomerase;
Vascular endothelial cell;
X-irradiation;
Cell survival.

INTRODUCTION

A telomere is a structure consisting of thousands of repeats and accessory peptide factors located at the termini of human chromosomes[1,2]. Telomeres become shortened gradually because of the incomplete DNA duplication at the chromosome ends at each cell cycle. Such telomere shortening has been observed in peripheral blood nuclear cells with aging[3-5]. The elderly have shorter telomeres in their somatic cells. Telomere shortening is accelerated by physical and mental stress with disease conditions[6-13].

X-ray exposure has been reported to induce cytopathological effects. X-irradiation to induce telomere-associated cell senescence among different kinds of transformed immortal cells derived from fibroblasts,

lens epithelial cells, endothelial cells, chondrocytes or keratinocytes[14-18]. All these previous reports concerning to telomere conditions affected by X-irradiation have described about cancer cells or immortalized cells. In addition, vascular endothelium has not been well analyzed in terms of X-ray-induced genotoxic effects including telomeric changes.

The aim of the present study was to elucidate how the different doses of X-irradiation affect the telomeric features including the telomere length, and the function and the expression of telomere-associated components (TERT, TERC, TRF1 and TRF2) of non-transformed vascular endothelial cell *in vitro*.

MATERIALS AND METHODS

Cell culture

Human umbilical venous endothelial cells (HUVEC) were purchased from Clonetics Corp. (San Diego, CA). They were cultured in endothelial cell growth medium (Clonetics Corp.). The cells were cultured at 37 ! and 5% CO_2 in a gelatin-coated flask (Iwaki Glass, 2 Chiba, Japan), and routine subcultivation was done every 2 days with a split ratio of 1:4, and used at the third passage. Cells were counted at this stage. They were X-irradiated 2Gy/min. On day 3 after the irradiation, the cells were collected and subjected to further analyses. Cells were counted using a hemocytometer. Population doublings (PDs) were calculated using the formula: PD=[log (expansion)/log 2], where expansion was the number of cells harvested divided by the initial number of cells seeded.

X-irradiation

X-rays were delivered from a soft X-ray generator (SOF-TEX M-150WE, Japan) operating at 100kVp and 3.5mA. The cells in the cell culture dishes were placed on an irradiation stage 30cm from the radiation source. The corresponding dose rate was 2Gy/min.

Senescence-associated β-galactosidase (SA-β -Gal) expression

The cells were washed in PBS, fixed for 10min at room temperature in 2% formaldehyde/0.2% glutaraldehyde, rinsed in PBS, and incubated at 37 □ (no CO_2) with fresh SA-β-Gal staining solution. The staining solution was made up as follows: 1mg X-gal, per ml solution, 5mM potassium ferricyanide, 5mM potassium ferrocyanide, 2mM $MgCl_2$, 150mM NaCl, and 40mM citric acid and sodium phosphate at pH 6.0. This solution was left on the cells for 12 h to achieve the maximum staining. Hundred cells were scored from each well (plate) using a light microscope.

Telomere detection

Telomere detection was performed as previously described[4]. Restriction enzyme *Msp*I was used. *Msp*I recognizes and cuts tetranucleotide CCGG. Briefly, blood cell DNA was extracted from samples using and the DNA (0.1μg) were digested at 37°C with 1U *Msp*I for 2h. The digests (10μl) were resolved by agarose gel-electrophoresis, and transferred by Southern blotting to a positively charged nylon membrane (Roche Diagnostics, Mannheim, Germany). The blotted DNA fragments were hybridized to a hyper-sensitive probe of 500bp long (TTAGGG)n labeled with digoxigenin. The membrane was then incubated with anti-digoxigenin-AP-specific antibody. The telomere probe was visualized by CSPD (disodium 3-(4-methoxy-spiro {1,2-dio-xetane-3,2'-(5'-chloro) tri-cyclo [3.3.1.1] decan} -4-yl) phenyl phosphate) (provided with the kit). The membrane was then exposed to Fuji XR film with an intensifying screen. The smears of the autoradiogram were captured on an Image Master, and the telomere length was then assessed quantitatively. Every sample was measured in triplicate.

Terminal restriction fragment (TRF) length analysis

Telomere length distribution was analyzed by comparing the telomere length using a telomere percentage analysis with three intervals of length as defined by a molecular weight standard, *Hin*d III-digested λ phage DNA, as previously described[4]. The intensity (photo-stimulated luminescence: PSL) was quantified as follows: each telomeric sample was divided into grid squares as follows according to the molecular size ranges: >9.4, 9.4≥≥4.4 and >4.4kb. The percent of PSL in each molecular weight range was measured (%PSL=intensity of a defined region-background×100/ total lane intensity-background). The percentage of PSL in each molecular weight range was measured (%PSL = intensity of a defined region – background x 100/

Regular Paper

total lane intensity - background). The mean TRF was estimated using the formula S(ODi - background)/ S(ODi - background/Li)[19], where ODi is the chemiluminescent signal and Li is the length of the TRF fragment at position i.

RT-PCR analysis

Total RNA samples were extracted using RNAzol B (Teltest). mRNAs for human TERT, telomerase RNA component (TERC), and TRF1 and TRF2 were determined by RT-PCR using a DIG detection system (Roche Applied Science). Each human cDNA was produced by RT-PCR according to each human-derived sequence, as follows: TERC forward primer: 5'-TCTAACCCTAACTGAGAAGGGCGTAG-3',

TERC reverse primer

5'-GTTTGCTCTAGAATGAACGGTGGAAG-3',

β-actin forward primer

5'-CCTTCCTGGGCATGGAGTCCT-3' and

β-actin reverse primer

5'-GGAGCAATGATCTTGATCTTC-3' were used according to the published human TERT-cDNA sequences[20,21]. The TERC mRNA level was normalized to the β-actin mRNA level in the same sample. The PCR products were directly synthesized from 2μg of total RNA isolated from each sample using the Superscript one-step RT-PCR system with Platinum *Taq* (Invitrogen) and gene-specific primers according to the recommendations provided by the supplier. The PCR products were analyzed by agarose gel electrophoresis (1.3%) followed by staining with ethidium bromide and scanning with Gel-Doc (Bio-Rad). For semiquantitative PCR, β-actin was used as an internal control to evaluate total RNA input, as previously described by our group[22].

Western blot and other analyses

Cells from a dish were homogenized with 100μl lysis buffer (100mM Tris pH 6.8, 4% SDS, 20% glycerol containing the protease inhibitor M phenylmethanesulfonyl fluoride, 0.1mM, leupeptin, 0.1μl, and aprotinin, 0.1μl). Gel electrophoresis was used to separate 10μg protein on a 10% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membranes (162-0112, Bio-Rad Labo-ratories, Hercules, California) blocked with 5% dry milk or blocking solution for Western blot (Roche). Membranes were blocked and incubated with antibodies against telomerase reverse transcriptase (TERT) (Rockland), TRF1 (Imgenex), TRF2 (Cell Signaling), or β-actin (Santa Cruz Biotechnology). Detection was performed with secondary horseradish peroxidase-conjugated antibodies (Chemicon) and the ECL detection system as previously described[22].

Telomerase activity

Telomerase activity was examined by means of a modified telomerase repeat amplification protocol (TRAP) method[23] with TeloChaser (Toyobo, Osaka, Japan) according to the manufacturer's instructions. Briefly, the substrate oligonucleotide is added to 0.5mg protein extract. If telomerase is present and active, telomeric repeats (GGTTAG) are added to the 3' end of the oligonucleotide. After amplification, the PCR products were resolved on a 12% polyacrylamide gel, stained with ethidium bromide, and detected using a FLA 5000 system (Fuji Film, Tokyo, Japan). The intensities of the bands were quantified with Image J (NIH). According to the manufacturer's instructions, the telomerase activities were calculated and presented as TPG (Total Product Generated).

Statistical analysis

Assays were repeated three times and analyzed statistically. Intergroup comparisons were performed using an independent samples *t*-test and one-way ANOVA. Paired samples were compared using the paired *t*-test. Significance was defined as p-values of <0.05. Group data are expressed as mean ± standard deviation. Statistical analyses were performed using the SPSS 10.0 software package (SPSS, Chicago, IL).

RESULTS

The alteration of population doubling (PD) and the induction of cell senescence after X-irradiation

The PD of the HUVECs was assessed on day 3 of culture after X-irradiation. The PD appeared to decrease proportionally with the increasing dose of X-ray (Figure 1a). The decreased PD indicated that cell growth was suppressed by X-irradiation in a dose dependent manner. Senescence-associated β-galactosi-

Regular Paper

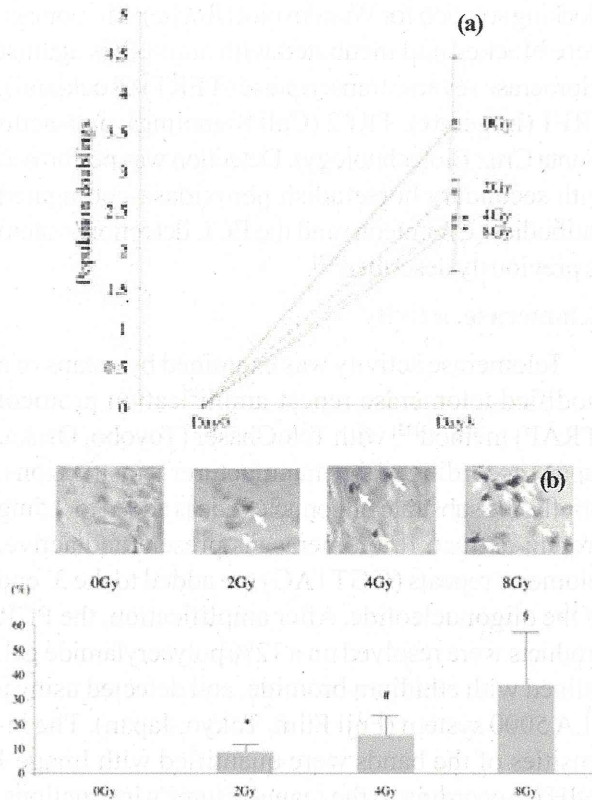


Figure 1 : The population doubling and the ratio of senescence-associated β-galactosidase (SA-β-Gal) staining of HUVECs cultured after X-irradiation; The PD after 3 days in culture. b. The upper panels show the SA-β-Gal-stained cells (white arrows) after different doses of X-irradiation. The lower panel shows the percentages of the cells that were stained. *p<0.05 vs. 0Gy. #p<0.05 vs. 2Gy. §p<0.05 vs. 4Gy.

dase (SA-β-Gal) expression became higher as X-ray dose increased (Figure 1b).

The alteration of the telomere lengths after X-irradiation

The mean TRF of the cultured HUVECs on day 3 was measured to see how much X-irradiation affected the telomeric DNA. Unexpectedly, the mean TRF level became longer within the lower doses of X-ray, 2 and 4Gy (Figure 2a). In the telomere length distribution, the longer telomeres were increasing and the shorter telomeres were decreasing after 2Gy- or 4Gy-X-ray irradiation (Figure 2b). This tendency disappeared in the case of 8Gy (Figure 2b).

The alteration of the telomerase activity after X-irradiation

The telomerase activity of HUVECs after 2Gy and 4Gy X-irradiation significantly decreased, however, did not changed after 8Gy irradiation (Figure 3).

Expression of telomere-associated RNA and proteins

The observation of telomere length change and the elevated telomerase activity in the cells led us to examine whether the expression of RNA and proteins associated with maintaining the telomere structure. The expression level of TERC did not change significantly after X-irradiation (Figure 4). The TERT protein expression tended to increase only after 4Gy irradiation, and the expression level after 8Gy irradiation was similar to

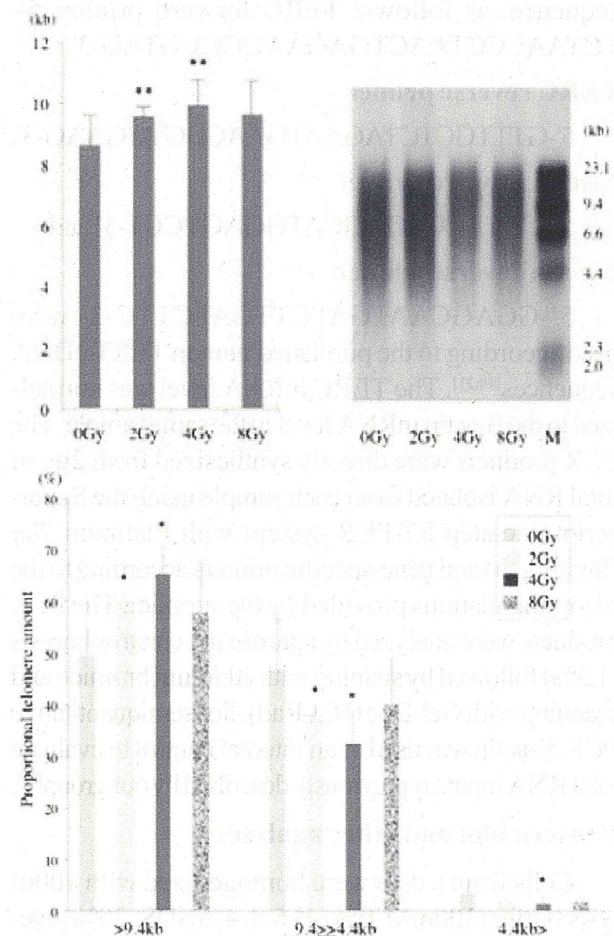


Figure 2 : The mean telomere length and the telomere length distribution of HUVECs after X-irradiation; The mean telomere lengths and a representative genomic Southern blot result with telomere DNA probe are shown. b. The telomere length distribution. The horizontal bars represent the standard deviation. The *Msp*I-terminal restriction fragment lengths are presented as the mean values ± standard deviation. The horizontal bars represent the standard deviation.

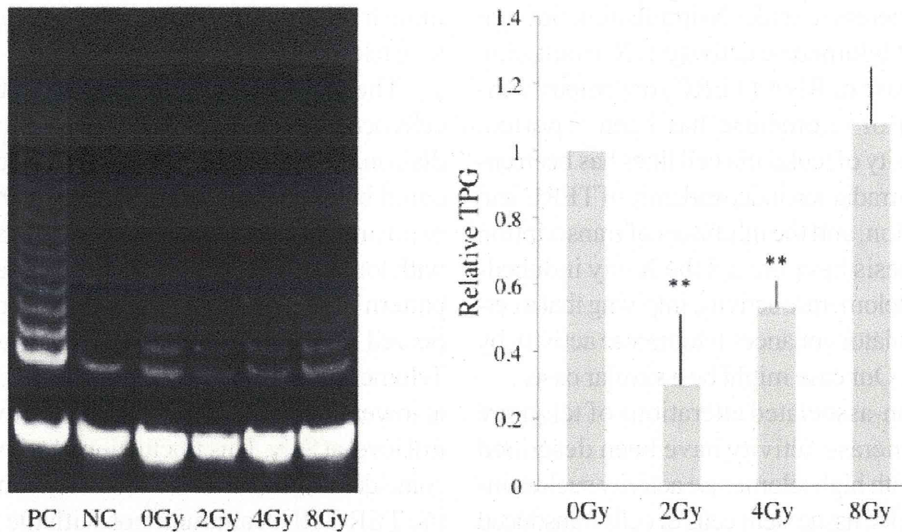


Figure 3 : The telomerase activity of endothelial cells after X-irradiation; The left panel shows the telomerase activity at different doses of X-irradiation as the TPG (Total Product Generated) levels. The TPG is presented as a proportional ratio of a ladder density of a sample to that at 0Gy. The left panel shows a photograph of representative TRAP assay result for HUVECs after X-irradiation. The materials used for the positive control (pc) and negative control (nc) were provided with the kit. *p<0.05 vs. 0Gy

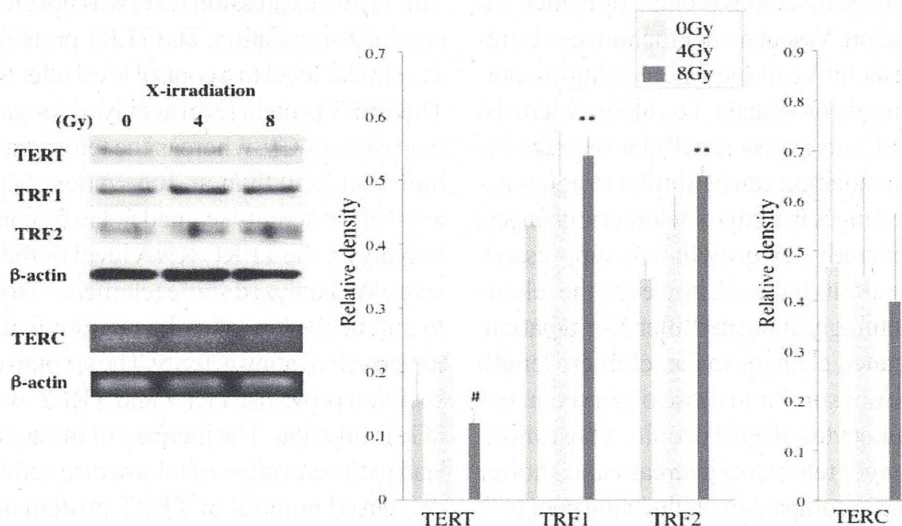


Figure 4 : The expression level for telomerase RNA component (TERC) and the expression levels of telomere-associated proteins, TERT, TRF1 and TRF2. Representative (left) and summarized (right) results for the mRNA expression (semiquantitative RT-PCR) of TERC and the western blot analysis of telomere-associated proteins (TERT, TRF1 and TRF2) after X-irradiation are shown. The relative expression levels were determined compared to that of β-actin (set at 1). Horizontal bars represent standard deviations. n=6. ** p<0.01 vs. 0Gy, #p<0.05 vs. 4Gy

the original level at 0Gy. On the other hand, the protein expressions of TRF1 and TRF2 increased significantly after 8Gy irradiation.

DISCUSSION

In the present report, telomerase activity did not coincide to the level of RNA or protein expression of telomerase components, either. Unexpectedly restored telomerase activity under X-irradiation was observed, and it was accompanied by both of the increased levels of TRF1 and TRF2. This implies the possibility that the levels of accessory components for telomerase activation, including not only TRF1 and TRF2 possibly but

also others, are increased under X-irradiation. Such an up-regulation of telomerase activity at X-irradiation without an increase of RNA (TERC) or protein component (TERT) of telomerase has been reported. Telomerase activity of leukemia cell lines has been enhanced under X-irradiation independently of TERC and TERT transcription, and the inhibitors of transcription or protein synthesis have erased the X-ray induced-up-regulation of telomerase activity, implying that a certain positive regulator enhances telomerase activity by X-irradiation[24]. Our case might be a similar case.

The radiation-associated alterations of telomere length and telomerase activity have been described mainly in cells with high telomerase activity such as including cancer cells, tissue stem cells or cells transduced with a TERT expression vector[25-29]. In the present study, a non-transformed somatic cell, HUVEC, was used to analyze telomere-associated alterations induced by X-irradiation. Aging-associated genomic changes contain X-ray-induced mutations brought by natural environmental radiation. Vascular endothelium can be regarded as a representative tissue where aging-associated somatic change is prominent, i.e., atherosclerosis. We chose HUVECs to assess if cellular damage induced by ionizing radiation can be similar to aging-associated cellular changes in terms of telomeric changes.

In the present study cell growth was suppressed, and cell senescence was induced, however, the telomere length was seemingly elongated after X-irradiation. The X-ray-associated changes in the telomere length distribution were not similar to those observed in peripheral blood leukocytes of aged people, whose mean TRF shortens, longer telomeres decrease and shorter telomeres increase in comparison with young people[4]. From this view, the survived cells seemingly bore rather younger patterns of the telomere length distribution than non-irradiated control cells. Moreover, the mean telomere length did not coincide to the telomerase activity, and telomere elongation was observed even with telomerase activity lowered by X-irradiation. Together with the reduction of the proportional amount of short telomeres, the apparent X-ray-associated-elongation of telomere length was not likely induced by the activation of telomerase but by loss of cells bearing short telomeres. Cells bearing short telomeres were old cells having experienced many cycles of mitosis, which were more fragile to X-irradiation than young cells bearing long telomeres.

The present results suggested that the endothelial cells bearing long telomeres could survive after X-irradiation, and the cells bearing short telomeres, which could be regarded as old cells, were fragile to X-ray exposure. However, the X-ray resistance of the cells with long telomeres was lost at 8Gy irradiation, as the pattern of the telomere length distribution of 8Gy appeared to return to a pattern similar to that of 0Gy. Telomerase activity was suppressed after X-irradiation at lower doses, 2Gy and 4Gy, and recovered to a control level at 8Gy. This fluctuation of telomerase activity coincided neither with the TERT RNA amount, nor with the TERC RNA amount, nor with the TERT protein level. It seems that a lower dose of X-ray suppressed the cell growth and lowered the telomerase activity of most cells, and at a higher dose only cells with a potential to induce higher telomerase activity could survive. The TERC expression level was not changed after any dose of X-irradiation. The TERT protein level was kept at a similar level to a control level after 8Gy irradiation. The TERT protein level at 8Gy was significantly lower than that at 4Gy, whereas the telomerase activity was higher at 8Gy than at 4Gy. After X-irradiation, the telomerase activity seemed not to be controlled quantitatively by the TERC RNA level or the TERT protein level. We analyzed some telomere-associated proteins to pursue the hints for the paradoxically elevated telomere-elongating activity. The amount of telomere-associated proteins, TRF1 and TRF2, were elevated at 8Gy irradiation. The increase of these co-factors might lead to the elevation of telomerase activity even with a decreased amount of TERT protein at 8Gy. The increases of TRF1 and TRF2 might be induced by cellular protective mechanism in jeopardized cells, so-called hormesis effects. TRF1 has been reported to negatively control the telomerase-associated telomere length maintenance[30]. Under telomere-erosive conditions, however, TRF 1 can contribute to telomere stability[31,32]. TRF2 is associated with stabilizing the telomere structure[33,34]. The TRF1 and TRF2 expression levels were maintained within the normal or elevated to a higher range after X-irradiation. While the X-ray-irradiation seemed to impair the telomere length maintenance, the expression of TRF1 and TRF2 may contribute to

Regular Paper

telomerase activation possibly with other telomere- or telomerase-associated factor(s) rather for cell survival than elongating telomeres. Our observation of the paradoxical enhancement of the telomerase activity may therefore be a pivotal step for the understanding of cell protection under genotoxic condition with X-irradiation. Somatic cells with highly elevated telomerase activity can survive through X-ray exposure, but may lead to tumorigenesis at a later stage[35,36]. The cellular mechanism(s) responsible for the protective enhancement in the telomerase activity of somatic cells injured by X-ray irradiation will need to be elucidated in further studies. Alterations in the behaviors of the telomere structure-associated components in vascular endothelial cells exposed to X-ray also warrant further investigation.

ACKNOWLEDGMENT

We would like to thank Ms. K. Tsuchida, Ms. S. Taguchi, and Ms. Y. Ueda for their expert technical assistance. This work was supported by grants from the Ministry of Education, Science, and Culture of Japan (#23590885), the 2012 Health and Labour Sciences Research Grants Comprehensive Research on Life-Style Related Diseases including Cardiovascular Diseases and Diabetes Mellitus, and the National Natural Science Fund (NSFC) (81170329/H2501).

REFERENCE

[1] E.H.Blackburn; Nature (London), **350**, 569-573 **(1991)**.
[2] V.A.Zakian; Science, **270**, 1601-1607 **(1995)**.
[3] H.Iwama, K.Ohyashiki, J.H.Ohyashiki, S.Hayashi, N.Yahata, K.Ando, K.Toyama, A.Hoshika, M.Takasaki, M.Mori, J.W.Shay; Human Genetics, **102**, 397-402 **(1998)**.
[4] J-Z.Guan, T.Maeda, M.Sugano, J.Oyama, Y.Higuchi, N.Makino; Molecular Cellular Biochemistry, **304**, 253–260 **(2007)**
[5] G.Aubert, P.M.Lansdorp; Physiological Review, **88**, 557-579 **(2008)**.
[6] K.Okuda, M.Y.Khan, J.Skurnick, M.Kimura, H.Aviv, A.Aviv; Atherosclerosis, **152**, 391-398 **(2000)**.
[7] S.Brouilette, R.K.Singh, J.R.Thompson, A.H.Goodall, N.J.Samani; Arteriosclerosis Thrombosis Vascular Biology, **23**, 842-846 **(2003)**.
[8] E.S.Epel, E.H.Blackburn, J.Lin, F.S.Dhabhar, N.E.Adler, J.D.Morrow, R.M.Cawthon; Proceedings of National Academy of Science of USA, **101**, 17312-17315 **(2004)**.
[9] A.M.Valdes, T.Andrew, J.P.Gardner, M.Kimura, E.Oelsner, L.F.Cherkas, A.Aviv, T.D.Spector; Lancet, **366**, 662-664 **(2005)**.
[10] O.Uziel, J.A.Singer, V.Danicek, G.Sahar, E.Berkov, M.Luchansky, A.Fraser, R.Ram, M.Lahav; Experimental Gerontology, **42**, 971-978 **(2007)**.
[11] L.A.Panossian, V.R.Porter, H.F.Valenzuela, X.Zhu, E.Reback, D.Masterman, J.L.Cummings, R.B.Effros; Neurobiology of Aging, **24**, 77-84 **(2003)**.
[12] J.Z.Guan, T.Maeda, M.Sugano, J.Oyama, Y.Higuchi, T.Suzuki, N.Makino; Journal of Gerontology A Biological Science and Medical Science, **62**, 1199-1203 **(2007)**.
[13] J.Z.Guan, T.Maeda, M.Sugano, J.Oyama, Y.Higuchi, T.Suzuki, N.Makino; Journal of Gerontology A Biological Science and Medical Science, **63**, 467-473 **(2008)**.
[14] J.P.de Magalhães, F.Chainiaux, F.de Longueville, V.Mainfroid, V.Migeot, L.Marcq, J.Remacle, M.Salmon, O.Toussaint; Experimental Gerontology, **39**, 1379-1389 **(2004)**.
[15] K.Yudoh, Y.Nguyen, H.Nakamura, K.Hongo-Masuko, T.Kato, K.Nishioka; Arthritis Research and Therapy, **7**, R380-391 **(2005)**.
[16] X.Q.Huang, J.Wang, J.P.Liu, H.Feng, W.B.Liu, Q.Yan, Y.Liu, S.M.Sun, M.Deng, L.Gong, Y.Liu, D.W.Li; Investigative Ophthalmology and Visual Science, **46**, 2503-2513 **(2005)**.
[17] J.Duan, J.Duan, Z.Zhang, T.Tong; International Journal of Biochemistry and Cell Biology, **37**, 1407-1420 **(2005)**.
[18] N.Büchner, T.C.Zschauer, M.Lukosz, J.Altschmied, J.Haendeler; Experimental Gerontology, **45**, 558-562 **(2010)**.
[19] T.de Lange, L.Shiue, R.M.Myers, D.R.Cox, S.L.Naylor, A.M.Killery, H.E.Varmus; Molecular and Cellular Biology, **10**, 518–527 **(1990)**.
[20] M.Fujiwara, H.Kamma, W.Wu, Y.Yano, S.Homma, H.Satoh; International Journal of Oncology, **29**, 445-451 **(2006)**.
[21] Q.Tao, B.Lv, B.Qiao, C.Q.Zheng, Z.F.Chen; Oral Oncology, **45**, e239-244 **(2009)**.
[22] M.Sugano, K.Tsuchida, T.Maeda, N.Makino; Ath-

erosclerosis, **191**, 33–39 **(2007)**.

[23] T.Nagata, Y.Takada, A.Ono, K.Nagata, Y.Konishi, T.Nukina, M.Ono, A.Matsugami, A.Furukawa, N.Fujimoto, H.Fukuda, H.Nakagama, M.Katahira; Nucleic Acids Research, **36**, 6816–6824 **(2008)**.

[24] P.Finnon, A.R.Silver, S.D.Bouffler; Carcinogenesis, **21**, 573-578 **(2000)**.

[25] J.Huang, Y.X.Bai, S.W.Han, S.S.Ng, D.D.Jing, B.C.Wong, C.F.Huang, H.F.Kung, M.C.Lin; Biochemical and Biophysical Research Communication, **301**, 627-632 **(2003)**.

[26] Y.Katakura, K.Yoshizaki, T.Yasuda, T.Tsunematsu, N.Uehara, T.Miura, T.Fujiki, S.Shirahata; Biochemical and Biophysical Research Communication, **334**, 450–459 **(2005)**.

[27] R.Sheng, Z.L.Gu, M.L.Xie, W.X.Zhou, C.Y.Guo; European Journal of Pharmacology, **641**, 199–206 **(2010)**.

[28] E.Nitta, M.Yamashita, K.Hosokawa, M.Xian, K.Takubo, F.Arai, S.Nakada, T.Suda; Blood, **117**, 4169-4180 **(2011)**.

[29] I.R.Indran, M.P.Hande, S.Pervaiz; Cancer Research, **71**, 266-276 **(2011)**.

[30] B.van Steensel, T.de Lange; Nature (London), **385**, 740-743 **(1997)**.

[31] C.Y.Soohoo, R.Shi, T.H.Lee, P.Huang, K.P.Lu, X.Z.Zhou; Journal of Biological Chemistry, **286**, 3894–3906 **(2011)**.

[32] M.A.McKerlie, S.Lin, X.D.Zhu; Nucleic Acids Research, **40**, 3975-3989 **(2012)**.

[33] B.van Steensel, A.Smogorzewska, T.de Lange; Cell, **92**, 401-413 **(1998)**.

[34] P.Zhang, C.Dilley, M.P.Mattson; Neuroscience, **145**, 1439–1448 **(2007)**.

[35] A.Chebel, J.P.Rouault, I.Urbanowicz, L.Baseggio, W.W.Chien, G.Salles, M.Ffrench; Journal of Biological Chemistry, **284**, 35725-35734 **(2009)**.

[36] N.Miura, I.Horikawa, A.Nishimoto, H.Ohmura, H.Ito, S.Hirohashi, J.W.Shay, M.Oshimura; **93**, 56-62 **(1997)**.